SPERM ACTIVATION BY ARBACIA EGG EXTRACTS, WITH SPECIAL REFERENCE TO ECHINOCHROME

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It has long been known that the eggs of various marine invertebrates secrete substances which markedly affect the behavior of sperm. One has merely to rinse eggs in sea water and add this water to a sperm suspension to produce striking changes. These effects are classified by Lillie (1924) as activation, aggregation, and agglutination. Activation is a stimulation of the sperm, bringing them instantaneously from an inactive state (as in the testis) to a high pitch of activity. Aggregation constitutes the gradual accumulation of the sperm within a region of high concentration of various agents, and in the case of egg-secretions, appears to be a chemotaxis. Agglutination is the clumping of the sperm exposed to egg-secretions. While some properties of the substances active in egg-secretions are known (Tyler and Fox, 1940), the substances themselves have not been isolated from eggs in pure enough form that we can attribute these properties to definite chemical entities. In 1939, Hartmann, Schartau, Kuhn, and Wallenfels reported that echinochrome, the pigment which gives *Arbacia* eggs their reddish color, produces the same stimulation in *Arbacia pustulosa* sperm as does the egg-secretion itself, and is effective in dilutions as great as 1 : 2,000,000,000. An attempt was made to duplicate these results, using *Arbacia punctulata* sperm, and crystalline echinochrome kindly supplied by Dr. E. G. Ball, which he had isolated from *A. punctulata* eggs (1934). No stimulating effect could be detected. Subsequently there appeared a fuller account by Hartmann and Schartau (1939), and a report by Tyler (1939) of negative results with *Strongylocentrotus purpuratus*. Because of this, it seemed worthwhile to repeat and extend the experiments with *A. punctulata*.

Echinochrome as the Activator

In preparing the solutions, both sea water and isotonic sodium chloride were used. In sodium chloride, the sperm do not agglutinate, which

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sometimes facilitates comparison of sperm activity. The egg-secretions were obtained by suspending one volume of eggs in 100 volumes of sea water (or NaCl) for a half-hour. The liquid, filtered free of eggs, is generally referred to as egg-water, and is extremely effective in bringing about activation and agglutination. The concentrations of echinochrome ranged from 1:2,500,000,000 to 1:25,000. At the latter concentration, the echinochrome solution is pink in color and therefore is well above the concentration of echinochrome in active egg-water, which was colorless. The activating properties of the echinochrome solutions were tested immediately after preparation, to avoid possible loss of the echinochrome through its precipitation as a calcium salt in sea water, or its decomposition in alkaline solutions.

Sperm respond readily to differences in pH. Therefore, in testing potency of echinochrome, particular attention was given to the control of pH in all solutions used. Glycyl-glycine (.001 M) and piperazine (.001 M) were used as buffers in preference to phosphate, which tends to precipitate calcium and magnesium from the sea-water solutions (Tyler and Horowitz, 1937). In these experiments little strain is placed upon the buffer systems, and measurements with the glass electrode showed these low concentrations to be adequate. In any single series, the egg-water and echinochrome solutions, and the sperm suspensions were prepared with the same buffer and the pH was measured with a glass electrode before and after the activation tests were made, as further precaution against differences in hydrogen ion concentration. Most of the tests were carried out at a pH level where the sperm were inactive, but were readily activated when egg-water at the same pH was added. This pH value was found to be in the neighborhood of 6.0 for the sea water and 7.5 for the isotonic sodium chloride solutions. The actual pH values varied with the individual sea-urchin, and increased if the dry sperm was allowed to age. In these experiments the absolute value is not important, since in every case a control test with egg-water was made along with each test of echinochrome. Accordingly, if echinochrome is the activating agent in egg-water, it should show activating properties at the same pH as the egg-water.

Two methods of testing were employed. In one, the dry sperm, that is, the sperm taken directly from the testis with a minimum of moisture, was diluted to about 1:100 in buffered sea water or isotonic NaCl. A drop of this was covered with a cover-glass, and the egg-water and echinochrome pipetted into opposite sides of the drop. In this way the slightest response of the sperm could be detected and a precise comparison made between the two solutions. Adding dry sperm directly to the test solution sometimes gave more spectacular differences in re-

sponse, but where small differences are involved, the first method is freer from subjective interpretation.

In no case did echinochrome activate the sperm. Each test was accompanied by a test with egg-water at the same pH, in which activation did occur.

Additional tests were carried out at higher pH values to supply more nearly normal conditions for the sperm. The results are not as clear-cut as with inactivated suspensions, since differences in speed of sperm are hard to estimate. However, in no case could it be said that the echinochrome definitely produced an increase in motility greater than did mere dilution with buffered sea water, whereas stimulation by egg-water could usually be seen clearly.

Chemotaxis in Echinochrome

To check the reported chemotactic effect of echinochrome, a few tests were made to compare the migration of sperm up glass capillary tubes. Tubes of the same diameter were washed and filled with sea water, egg-water, and echinochrome solution. The ends were then inserted into buffered sperm suspension, and migration measured at various times. The results were so variable that none of the solutions could be said to be definitely chemotactic on the basis of these few trials. Variations in the alkalinity of the glass probably played some part, since the volume of solution was small in proportion to the surface of the tube, and the buffer capacity of the solutions was low. Under such conditions a shift toward alkalinity could occur and give an illusory chemotactic effect by merely speeding the progress of the sperm.

Activity of the Echinochrome-protein Complex

The complex which echinochrome forms with proteins from the *Arbacia* egg was reported by Kuhn and Wallenfels (1940) to have sperm-stimulating properties in even greater dilutions (1 : 300,000,000,000) than echinochrome alone, in uncombined form. A similar echinochrome complex was extracted from *Arbacia punctulata* by their method. Eggs were frozen, crushed, and extracted with sea water. After filtering, an equal amount of saturated ammonium sulphate solution was added, bringing down a rose-colored precipitate which redissolved in sea water without leaving any residue. It was purified by repeated precipitation, centrifugation, and decantation.

This complex, buffered and tested in the same way as echinochrome, both activated and agglutinated *punctulata* sperm. In biological properties and solubility, therefore, it is the same as the tertiary complex ob-

tained by Kuhn and Wallenfels. In view of the tests with pure echinochrome, however, the activity of the complex would seem to be centered in the protein moiety rather than in the echinochrome.

Separation of the Agglutinating and Activating Properties of Egg-water

To determine more of the nature of the activating agent, egg-water was dialyzed against sea water for a half-hour, then both fractions buffered and tested for activity. The dialysate stimulated without agglutinating, while the residue both stimulated and agglutinated sperm. Dialysis, then, can separate the agglutinating and activating agents. Distillates obtained by gently boiling each fraction also showed activating properties, but the activity disappeared shortly after the distillate was buffered to pH 6. The original, unboiled dialysate retained its activity 24 hours, beyond which it was not tested. Similarly, a distillate from a repeatedly precipitated and washed sample of the echinochrome-protein complex could stimulate, when unbuffered (pH 9.0), and lost its activity within an hour after it was buffered to pH 6.6. This disappearance of stimulating activity from the distillates suggests that the activating agent had been altered during distillation. Improved methods of separation will probably yield a stable stimulating fraction. At present, it is important that distillates of egg-water, of egg-water dialysate, and of the echinochrome complex are similar in that they contain an activating substance.

Discussion

The absence of visible response of *A. punctulata* sperm to echinochrome is in agreement with Tyler's investigations with *Strongylocentrotus purpuratus*. He found that echinochrome brought about no increase in oxygen consumption of the sperm or eggs. On the other hand, these results do not agree with the observations of Hartmann and Schartau on *A. pustulosa,* which was found to be extremely sensitive to echinochrome solutions. The difference in response of *A. pustulosa* and *A. punctulata* could be attributed to species difference, although this would make the similarity of *A. punctulata* to the more distantly related *Strongylocentrotus* appear somewhat anomalous. Another possibility is that Hartmann and Schartau did not control pH in their solutions, since it is not mentioned in any of the papers on *A. pustulosa*. Their results, particularly the activity of highly dilute solutions (1:2,500,000,000), suggest that the activation is due to the normal alkalinity of the sea water used as a solvent.

Whatever the final answer may be with regard to echinochrome, we

must search farther for the answer to the general problem of sperm-activation by egg-secretions. Echinochrome is limited in occurrence, even within the class *Echinoidea*. Moreover, the egg-secretions from pigmented eggs will stimulate sperm from unpigmented species, and vice versa (cf. Woodward, 1918, Table I).

In the *Arbacia* egg there is some sperm-activating substance which will dialyze through a collodion membrane. It can, then, be separated from the agglutinating substance with which it is closely associated, but which will not dialyze. However, the echinochrome-protein complex carried with it through seven precipitations the power to activate as well as agglutinate sperm. In view of the ease with which the activator dialyzes, one might well expect it to be washed completely free from the agglutinating substance. Tyler (1939) also reports that partial purification of agglutinin from the keyhole limpet does not free it from activating properties. This leads one to suspect that there may be two substances present which activate sperm: one closely attached to the agglutinating substance, and one easily separated from it. On the other hand, the activating properties of rough distillates from the egg-water dialysate and the partially purified echinochrome-protein complex is some evidence of similarity between the activating agents in both, but it does not prove identity.

Earlier work on extracts from echinoderm eggs offers a possible explanation of these observations. Woodward (1918) obtained, in addition to an ammonium sulphate precipitate of agglutinin, a barium chloride precipitate which showed lipolytic activity. Glaser (1921) pointed out that this lipolysin (and pancreatic lipase as well) could activate sperm. Yet, if this lipase is the activating agent in egg-water, it follows that the activator in the distillates must be some substance other than the lipase, since a protein would not distil. In this respect the work of Clowes and Bachman (1921) takes on added significance. They were not only the first to obtain sperm-activating distillates from egg-water, but also found that higher alcohols (propyl, allyl, and cinnamyl) and related substances activate sperm. Bringing these findings together, one might tentatively suggest that the immediately effective agent in sperm activation is an alcohol freed by the lipase. Then the presence of the alcohol or of the lipase would be adequate to produce sperm activation. It remains to be demonstrated that the sperm-activator that follows the agglutinating fraction is the lipase, and the dialysable, distillable activator is the product of the activity of that lipase. The hypothesis fits the framework of assembled facts, but substantiation will require considerable further investigation of egg-water fractions and egg extracts.

Summary

Echinochrome is not the agent in *A. punctulata* egg-water which stimulates sperm. The echinochrome-protein complex precipitated from the extract of crushed eggs by $(NH_4)_2SO_4$ is an effective sperm stimulator. From egg-water dialysate and from the echinochrome-protein complex a distillate can be obtained which has sperm-activating properties. It is tentatively suggested that a higher alcohol freed by a hydrolytic agent in the egg-water is the stimulating substance acting directly upon the sperm.

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